Inhibiting BMAA from Access to Decrease the Risk of Having Neurodegenerative Diseases

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Abstract

Purpose: Neurodegenerative diseases are commonly seen in groups that have greater age. Although the exact pathway of Alzheimer's is still unclear, the amino acid BMAA that Nostoc algae have fixed was predicted to inhibit PP2A proteins and cause Hyperphosphorylation on tau proteins and lead to tau protein tangle, which is the known agent that causes Alzheimer's. So, the paper is dedicated to inhibiting BMAA from inhibiting PP2A kinases and seeing whether it can control the rate of neurodegenerative diseases' appearance.

Keywords: BMAA, Parkinson, ALS, algae, PP2A, Tau protein, Western-blot therapy.

1. Introduction

Recently, cases of Amyotrophic diseases in younger people have brought attention back to the correlation between algae toxins and neurodegeneration diseases. Specifically, b-N-methylamino-l-alanine (BMAA), is a neurotoxin produced by Cyanobacteria of the genus Candida and can also be found at the roots of cycad plants. In the 1950s, the rate of Chamorro people who lived on Guam island and get infected by Parkinson's disease or the Amyotrophic lateral sclerosis increased to a level that is even higher than most of the developed countries by times.[1] Later research has shown that the habit of eating Megabats, who feeds on Cycas plants' seeds caused the accumulation of BMAA toxins inside residents at Guam island, and it was predicted that the similar structure between it and Alaine may fail to be recognized by the body and leftover inside the body. The residues of the BMAA have then caused damage to the Neuron system. And lead to the onset of diseases like ALS or Huntington's disease (HD).

Anatomy data have also proven the fact that BMAA toxins have accumulated in Alzheimer' patients' brains through the HPLC method by tagin 6-aminoquinolyl-N-hydroxysucc- inimidyl carbamate (AQC), a glowing amino acid to the infected brain and extract glowing samples that send to tests. [10] The result of that research has given out that several ALS and AD patients have got a significant level of BMAA concentration. And find BMAA toxins in all ALS and AD patients in their temporal lobe and frontal lobe. Which is also the functioning part for neurodegenerative diseases to emerge.

However, there is a vacancy in the study of the relationship between the potential of BMAA produced by Cyanobacteria; Algae bloom, or red tides occurs frequently on the east coast of China, and people of all ages tend to rely on water products that may get contaminated by Cyanobacteria including genus Candida caused by the pollution of chemical fertilizers and sufficient minerals brought up from the seabed attribute to water fluxes evocated by global warming.

This outburst of algae may threaten the health of humans who accidentally engaged and get contaminated algae accesses inside their bodies. Formal research papers have shown that rhesus macaques (an experiment choice of the simian family that have a high genetic degree of human similarity) experienced strong toxin reactions to BMAA including Muscle atrophy of the extremities, anterior horn cells of the spinal cord presence non-reactiveness, degradation of the cerebral cortex or pyramidal neurons, inactivation of Betz cells located at the cerebral and the unable of conductive ability inside the central neuron pathway.

Meanwhile, water bodies that are contaminated or have a higher level of BMAA toxin are wildly existed and may use as irrigation sources or even sources of tube water. Due to the boost of agriculture, the fertilizers that are rich in phosphate and fixed nitrogen polluted the water body. Usually, an aqueous environment is hard for the existing precipitations of phosphates or nitrogen due to their excellent solubility. But the leakage of fertilizers was well absorbed by the algae, thus helping them to reproduce frequently. And as houses take the place where plants were previously cultivated, water bodies like lakes or ponds have algae bloom more frequently as there are no other places for the nutrient flow. 35% of all testing samples near the living quarters have discovered different concentrations of BMAA toxin in America.[11]

From other research, another toxin called FHAB, which is also a byproduct of the red tide, is spares of govern,

the Oklahoma Senate Bill has noticed that entertainment places should be warning the concertation FHAB toxin. But ignored a regular test for the water body. Not to mention places that do not have related regulations at all.
Unfortunately, teenagers can get affected by BMAA in a smaller dose than adults, and the places where teenagers go for swim or travel usually have a significant level of BMAA contamination. In research locates in Aveiro in Portugal, Cerastoderma edul, a kind of cockle has been picked up continuously to test the toxin concentration after the filtration feeding.[8] The concentration of BMAA is significantly higher than the suggested safe level. And to another paper that focuses on tourism at Aveiro, the beaches and the swimming places are especially attractive to the teenagers. And many of the local dishes have the decoration of cockles; which need to be focused on seriously and treated timely.
So, this paper is focusing on proving the relationship between human attachment to algae-contaminated water bodies and neurodegeneration diseases. And this study gives early warning to teenagers who try to swim in ride tides to stop their risky attempt. And prevent fishermen to keep capture sea products in the contaminated areas, since toxins can accumulate through the food chain, to avoid the tragedy of Guam to occur again.

2. Methods

2.1. High-Pressure Liquid Chromatography

The testing of BMAA quantitively is through samples from the frontal cortex and temporal polar cortex in the brain. The examiner must take the parts of spinal cords from patients who've got ALS, Alzheimer's, and Huntington's disease. The method of blind analysis is used, and all samples should be tested at least two times to prevent the imprecision both artificially and physically. The brain tissue has been homogenized twice by 0.1 moles of trichloroacetic acid and centrifuged at 15800 g for 10 minutes to remove the free amino acids from the sample. The sample has been hydrolyzed overnight by 6 moles of HCl at 110 degrees. And freeze-dried the extractions, followed by testing the suspensions in the 100 II borate buffer for the existence of BMAA using the method of separation by C18 column.

2.2. G. catenatum cultivation[5]

The samples of the two algae are from the Center of Oceanography of the Faculty of Science, University of Lisbon. Where collected from the location of an offshore station near Maria Island, the samples have already experienced filtration through a series of glass fibers, activated charcoals, and a filter which has its smallest aperture of 0.22 μm. Autoclaved in Teflon bottles and adjusted salinity to 28 in order for further experiments.

2.3. PDS/ DMSO experiment

Using the method of double emulsion to fabricate SAHA-loaded PLGA/ DOTAP nanoparticles and create alternative ratios of these particles for the screening of the cytotoxicity evaluation. The PDS solution used must dissolve the detected number of SAHA into the DMSO solution by the proportion of diluting it with 11.5 times of water. The PDS solution then is introduced to the amino acid of BMAA, concluding for the results of inhibiting BMAA existence affect the rate of tau protein tanglings.

2.4. Western blot experiment

2.4.1 Exposure to antibodies: Using a stock of non-toxic antibodies for culturing the cells; The stock has to be dispersed through ultrasonic devices; The dispersed stocks then need to be diluted to a non-cytotoxic level; The conditional medium for cell cultivation should be removed and add the suspensions one just made; Using (DLS) dynamic light scattering to measure the particle distribution; Then wash the cells with PBS solution prepared formally.
2.4.2 Preparing the sample for the gel electrophoresis: Freezing and thawing the samples several times, sonicate the sample, then use solubilization buffer to direct lysis to extract proteins for western-blot experiment; Add protease inhibitor to prevent proteolysis. And use phosphatase inhibitors when needed, Extracting the sample under the temperature of 4 degrees. Centrifuge the sample and collect cell debris; add proteases inhibitor and put the lysates on ice to prevent proteolysis.
2.4.3 Electrophoresis: Use a concentration of 7-15% of acrylamide in the gel used for western blot; Fill the electrophoresis chamber and rinse the wells of the gel with the electrophoresis buffer; Add SDS samples at 95 degrees for three minutes long and then put in the environment of seventy-five degrees for ten minutes to denature the proteins in the sample; Load pre-stained marker and western marker into the empty well; Run the sample at constant voltage until the bromophenol blue added to the SDS sample buffer come to the bottom of the gel.
2.4.4 The gel after electrophoresis needs to be packed into an apparatus composed of one layer of membrane and two layers of filter paper, which helps the results to attach to the blotting pad; Add 5% of milk as the blocking solution to prevent non-specialized antibodies from binding; The results on the membrane then need to view under X-ray and evaluate the bands to determine the quantitively of this protein;

2.5. Purification of tau proteins by a chromatography [12]

The extracted tissue to form the human cell line is centrifuged twice under 23000g and lysed by using a French press (Sim- Amico). After passing through a 45um syringe filter, using the binding sites on the htau-GFP Ni^{2+}-binding6- His sites, the chromatography is processed using a Ni^{2+} column, the residue from the chromatography is then redissolved in the LB buffer and tested for the OD (occipital density) value and comparing with the curve from OD=0.5 to analyze the abundance of tangled tau proteins.

2.6. Extraction of tau protein-coding RNA and cultivation of normal tau proteins from human brain samples.

2.6.1 Isolate the tau protein RNA from the frontal cortex of the human brain samples using the guanidinium thiocyanate-phenol-chloroform.

2.6.2 Adding primer oligo- dT(2ng/ul).

2.6.3 Reverse transcribed by Superscript II reverse transcriptase under the buffering of M-MLV RT buffer (contains 200nM dNTPs and R Nasin for the substance of PCR).

2.6.4 PCR procedure is controlled by incubation at 25°C for 5 minutes, 30°C for 5 minutes; 37°C for 5 minutes and 42°C for 90 minutes; and 100°C for 5 minutes. PCR primers selected the rTAU1 tau-specific primers; Taq as polymerase and the site EcoRI was used in the amplification of RNAs. The human tau products are then digest sequencing by NdeI and EcoRI, the RNA complex is then inserted into the NdeI-EcoRI-digested pET28a prokaryote expression vector and introduced into the DH5 alpha E.coil. Where the tau protein RNA can then be functioned and keep producing human tau proteins for testing.

2.7. Tau protein Phosphorylation test.

Human tau proteins were added to the MOPS buffer, and 2mM of Lithium chloride was first added as the modulator of the experiment.

2.7.1 G3Kβ: adding 5uM of olomoucine to eliminate the effect of other proteins from the cdk5 group to affect the results of phosphorylation. 4 times Laemmili buffer is used to stop the phosphorylation process and the experiment tube should be put on ice to lower down the activity of G3Kβ.[6]

2.7.2 Isotopic marks make for the tests of phosphorylation on specific positions: γ32P-ATP were used as the contributing ATP source to see if the parts between serine 396 and serine 404 have been phosphorylated due to the fact that we knew this section is in charge of tau protein tangling.

3. Materials

3.1. Western blot preparation:

3.1.1. Solubilization buffer: Radio immunoprecipitation assay buffer (RIPA) containing 25mM Tris-Hcl, 150mM NaCl, 1% Nonidet P-40, 0.5-1% sodium deoxycholate, 0.1% SDS.

3.1.2 Protease inhibitors: 1mM phenylmethylsulphonyl fluoride (PMSF), 10μg/mL aprotinin, 5mM benzamidine, 20 μM
leupeptin, 10 μM pepstatin, and 5 mM EDTA.

3.1.3 Phosphatase inhibitors: 1mM p-nitrophenyl phosphate, 50 mM NaF, 1mM orthovanadate(Na_3Vo_4), and 20 nM calyculin A.

3.1.4 Polyacrylamide gel: SDS-PAGE gels, acrylamide, bis-methylene acrylamide solution, 0.1g/ mL $(NH_4)_2S_2O_8$ and TEMED ($(CH_3)_2NCH_2CH_2N(CH_3)_2$).

3.1.5 Power supply: Western blot apparatus (iBlot).

3.1.6 Electrophoresis buffer: 6 grams of TRIZMA-Base, 22.8 grams of glycine, and 1g of SDS to 1 liter of deionized water.

3.1.7 Transfer buffer: 12.1g TRIZMA-Base, 14.4 glycines in 950ml of deionized water, and then add 50mL of methanol.

3.1.8 Membrane paper filter: Polyvinylidene difluoride (PVDF) membranes in paper size.

3.1.9 Blocking solution: 2-3% bovine serum albumin (BSA) in PBS.

3.1.10 Antibodies: Dilute antibodies to 1/200- 1/1000 and peroxide (POD)-tagged secondary antibody to 1/1500-1/5000 by blocking solution.

3.1.11 Membrane wash: Tween 20 at a concentration of 0.05% to phosphate buffer.

3.1.12 Antibody signal detection: enhanced chemiluminescence (ECL) solution.

3.1.13 Stripping buffer: (62.5 mM Tris- HCl containing 2% SDS and 100 mM β- mercaptoethanol) to remove antibodies from the membrane after western-blot analyses.[7]

	Existance of BMAA	Activation of mglur5	Western-blot antibody test (Human cell line)	PDs/Dmso chromatography (Mice brain)
1. PP2A added	*	*	*	*
2.PP2A added	*	*	*	-
3.PP2A added	*	*	-	*
4.Anti-PP2A and PP2A added	*	*	-	-
5.PKA added	*	-	*	*
6.PKA added	*	-	*	-
7. PKA added	-	-	-	-
8.Anti-PKA and PKA added	*	-	-	-
9.G3Kβ added	*	N/A	*	*
10.G3Kβ added	*	N/A	-	*
11.G3Kβ added	-	N/A	-	-
12.Anti-G3Kβ and G3Kβ added	*	N/A	-	-

Explanations: (*) means the substance exists in this test or has a positive result for the tau protein test.
(-) means that the substance is not present in this test or has a negative result for the testing of tau protein existence.
(N/A) means that the receptor here is none related to the Microtubule Associated protein.

4. Results

4.1. Possible Result 1: BMAA introduced in the test with PP2A existed. When the mGluR5 receptor is inactive, both human cell line tested by western blot and mice hippocampal brain slice tested by chromatography results positive.

The BMAA has been introduced to the imitative human cell line and the brain slice of the mice, which both bind with the mGluR5 receptors and cause PP2A to dissociate with its subunit PP2Ac, which indirectly inhibits the activity of PP2A by phosphorylation of the Try307 site on PP2A when losing the regulation of this kinase, microtubule alpha, and beta over-phosphorylation and cause tau protein tangling.[9]

4.2. Possible Results 2 &3: BMAA has been introduced with PP2A together, and when the mGluR5 receptor is activated, only the western blotting results in a human cell line or chromatography result from the slice shows positive for the existence of tangled tau protein.

The BMAA has successively activated the mGluR5 receptor and released the PP2Ac parts from the PP2A kinase. The loss of phosphorite controlling factor in the neuron will soon cause hyperphosphorylation on tau proteins and tangles but is sufficient in the human cell line or in the mice's brain slice.

4.3. Possible Result 4: The presence of the Anti-PP2A inhibitor I1PP2A blocked the total process and won't cause any tau protein tangling.

When introducing BMAA with both PP2A and Anti-PP2As, although the mGluR5 receptor is activated, the PP2A won't be released from its subunits and there won't be a presence of tangled tau proteins in both the human cell line and the mice's brain slice/

4.4. Possible Result 5: Introducing BMAA into another phosphorylation-related kinase (PKA) can cause tau protein tangling in both human cell lines and brain slices without the participation of mGluR5.

BMAA was introduced with the substance of PKA, without the need for the mGluR5 pathway, the tau proteins in the human cell line and the mice brain piece can be both hyperphosphorylated and tangled.

4.5. Possible Result 6: Introducing BMAA with PKA can only hyperphosphorylated tau proteins in the human cell line but not in the mice's brain piece.

The introduced PKA kinase protein can bind with tau proteins tightly whenever times the microtubule folded and keep phosphorylated the tubulins, and only cause tau protein GFP overexpression and is detected by the western blot.

4.6. Possible Result 7: The absence of BMAA in the experiment cannot allow PKA to hyper-

phosphorylate the tau proteins.

Although the pathway of mGluR5 is not functioning for PKA, without the attendance of BMAA other kinases also can't phosphorylate tau proteins.

4.7. Possible Result 8: BMAA is introduced into a condition that contains both PKA substance and Anti PKA, and no results for tau protein tangling in both human cell line and the brain slice.

A dependent pathway on BMAA for another kind of kinase is found and it won't do any change when the presence of its anti-molecular substance.

4.8. Possible Results 9 &10: After introducing BMAA into G3KB, tau protein tangling is constantly found in test objects, of the mice's brain slice.

The existence of G3KB binding to microtubules and the isotopes on it helps to identify the size of the tau fragment under the chromatography.

4.9. Possible Results 11 & 12: When BMAA is absent or the Anti-proteinase substance has appeared, the process of over-phosphorylation is regulated and stopped from tau protein tangling.

Adding Anti-G3KB or lack of BMAA disables the microtubule assistant proteins to bind with different tubulins, thus won't transfer the isotopes marked on the binding site of the modified G3KB molecules.

5. Discussion

The series of experiments were designed to test the following questions: First, is PP2A inhibition by BMAA activating mGluR5 the majority reason why human tau proteins tangled. Second, does the process of activating mGluR5 receptors necessary in all different ways of phosphorylation of tau proteins. Third, can BMAA activate microtubule-associated proteins directly instead of indirect inhibition or playing the role of inducive -coordinator.

Based on the first question, the first four questions are designed based on the introduction of PP2A in the system, and based on the known materials, to furtherly predict whether this theory can be proven in practice in vitro experiments. The possible result 1 obeys what I've initially indicated, the PP2A is fundamental in the hyper-phosphorylation of tau proteins, and because the western-blot experiment has tested positive for the Tau-5 and 5A6 immunoglobins, also the chromatography shows that Ni^{2+} sites have been bonded, so we can indicate that human and animal tau proteins are homogeneous for their formation.

For the possible result 2, it is more likely to be accepted by the current research papers, results have shown in different papers that inhibited PP2A kinase can cause alpha and beta tubulins to be hyper-phosphorylated, and the tangled tau proteins shared the characteristics since those two tubulins are the substance for protein tau.[13] The second conclusion has been tested on Guan Island Alzheimer patients' brain slices, and strongly indicated that PP2A is the leading factor for Guan's cases.

Reversely, possible result 3 is an antagonist with the possible result 2, which indicates that chromatography tests for animal brain slices are more likely to show the appearance of tau protein. This hypothesis is also convincing to some extent. Independent research has shown that with the time of centrifugation of tau proteins and depolymerizing the proteins, random folding might occur which lowers the likelihood for PP2A to affect the phosphorylation level of the tau proteins, may also indicate that the process of transferring tau protein RNA into E.coli may be any uncertainty in this experiment that causes tau proteins to change its binding sites, and enable PP2A from binding it, which lead to the non-observation of the western-blot experiment in the human cell line test.

The possible result 4 is an indicator of the importance of PP2A in causing tau protein tangling and a negative experiment group. The anti-PP2A inhibits the PP2Ac subunits from dropping off even if the mGluR5 is activated, so the phosphorylation level of tau proteins is still controlled under standard level, which also echoes the hypothesis of PP2A playing an important role in controlling tau protein tangling.[4]

The possible results 5 and 6 are the conclusion from previous papers and result 5 is a further indication. The substance PKA is selected for two reasons, firstly, it is another kind of kinase that existed inside the axons of mammals, and is not related to the mGluR5 receptor, so it can prove if BMAA can affect kinase through other pathways.[14] Secondly, according to the concern of transferring tau protein RNA into E. coli might change the position of the phosphorylation sites, PKA has the highest value of nearly 1 in the test of the ratio between the binding rate for the original tau proteins and tau proteins formed after centrifugation twice. So, using it also can eliminate the uncertainties caused by the inevitable inaccuracies in the experimental methods. The possible result 5 follows the results that it will not affect by the times of folding in tau protein. But the possible result 6 reflexes that there are other factors or differences between human and mice's tau protein. Which can be discovered in further experiments.

Possible results 7 and 8 independently show the

relationship of BMAA with PKA and PKA with tau protein tangling. In the possible result 7, even if the PKA kinase exists in the system, without the over-activation of BMAA neurotoxin, there still will not be abnormal tau proteins formed. And possible result 8 is more likely to show that in this pathway, PKA kinase is the dependent variable, and at the initiation of this process, a single presence of BMAA can't cause any damage to tau proteins.

The series of possible results 9 to 12 is a parallel experiment using microtubule-associated proteins instead of using phosphorylation enzymes. This is due to the purpose of locating where exactly BMAA is functioned to lead to the result of tau protein tangling. Is it can only affect the catalysts of tau protein formation or it can also affect the 'raw materials' of tau protein synthesis?

The G3Kβ molecule is already known that can strongly affect tau protein synthesis in the Bovis's brain slice.[9] So the goal of the experiment is to discover whether this effect is still functioning inside the human cell line. The possible result 9 answered positive and the possible result 10 shows negatively. Both results are not supported by any previous experiments so it's unlikely to predict the possibility of each process functioning.

The possible results 11 and 12 have similar functions to possible results 7 and 8. It is designed to confirm that G3Kβ can't affect tau protein tangling without the existence of BMAA and is the functioning substrate of tau protein hyper-phosphorylation. Both results are likely to be attained and accordant with my hypothesis.

6. Conclusion

From the experiments of PP2A, a negative experiment group of anti-PP2A, and parallel experiments of PKA and G3Kβ , it can be conclude that PP2A is affected by BMAA through activation of the mGluR5 post-synaptic receptor, which causes PP2A to detach with tau proteins when it's forming. And it also causes extra phosphorate groups to be added to the section of Ser 262 and Ser 356, which cause hyper-phosphorylation and tau protein tangling. PP2A pathway is the majority pathway for BMAA to affect the prevalence of Alzheimer's disease in mammals. And further experiments can be done focusing on the pathways with how BMAA affects PKA and G3Kβ molecules. And to find the cure of Alzheimer's by controlling kinases and microtubules in average phosphorylation level.

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